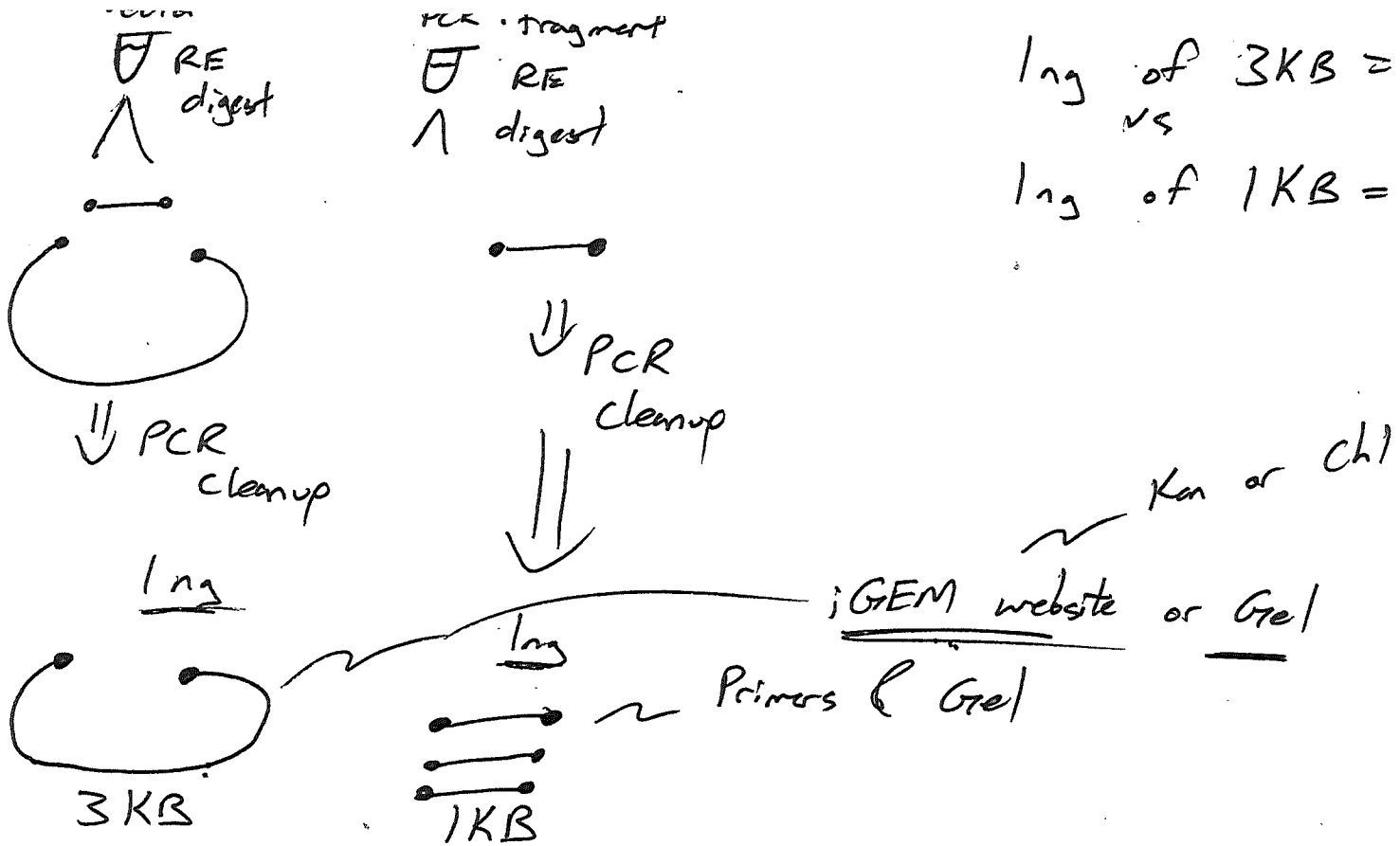
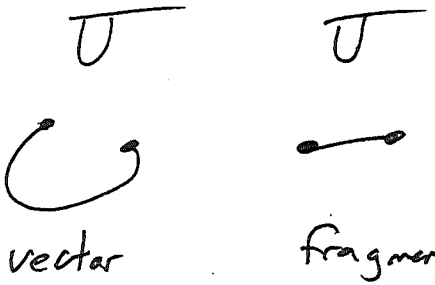


1ng of 3KB = 1x
vs
1ng of 1KB = 3x

[DNA] what ratio 1:3 or 1:5 of vector to insert (molar ratio)

~~X KB~~ x

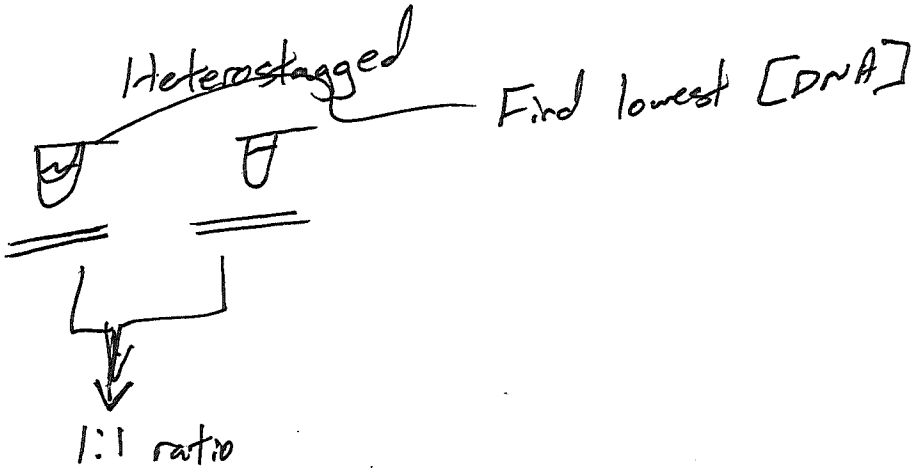
X bp x $\frac{\text{mol}}{650\text{-}660 \text{ amu per bp}}$



1 ug - 2 ug of DNA for digestion

pBBRIMCS.2

iGEM
- Kan
- Chl



06 Jun 2018

Competent cells procedure:

Observations

Made 1M stock/L NaOAc
- pH 7.01

Created CCMB80 buffer
- pH 6.41

Creates SOB
- pH 7.50

8 tubes

4 PCR products each in tetr + Headlimpeyn

Artificial Sea water

4	2	Per Liter →
		800 mL Deionized Water
110	55	27.5 g NaCl
15.2	7.6	3.8 g $MgCl_2 \cdot 6H_2O$
27.12	13.86	6.78 g $MgSO_4 \cdot 7H_2O$
2.88	1.44	0.72 g KCl
2.48	1.24	0.62 g $NaHCO_3$
11.16	5.58	2.79 g $CaCl_2 \cdot 2H_2O$
4	2	1 g NH_4Cl
2	0.10	0.05 g K_2HPO_4
4 mL	2 mL	1 mL Wolfe's Trace Mineral Soln.

bring to 1 Liter w/ Deionized H_2O

PH ≈ 6.1 – 6.5

mmol/kg → mM $\frac{\text{m moles}}{\text{Liter}}$

~~1.035 kg Sea H_2O → 1~~

1.025 kg = 1L Sea water

$$1 \frac{\text{mmol}}{\cancel{\text{kg}}} \Big| \frac{1.025 \cancel{\text{kg}}}{1\text{L}} = 1.025 \text{ mMol/L}$$

Concentration in (mM)

Na^+	K^+	Cl^-
481.75	10.455	563.75

$$Na^+ \quad \frac{470 \text{ mmol}}{\text{kg}} \Big| \frac{1.025 \text{kg}}{1\text{L}} = 481.75 \text{ mM}$$

$$K^+ \quad \frac{10.2 \text{ mmol}}{\text{kg}} \Big| \frac{1.025 \text{kg}}{1\text{L}} = 10.455 \text{ mM}$$

$$Cl^- \quad \frac{550 \text{ mMol}}{\text{kg}} \Big| \frac{1.025 \text{kg}}{1\text{L}} = 563.75 \text{ mM}$$

Making chloramphenicol plate

1) get 1 15mL Sterile tube.
2) weigh out 50mg of chloramphenicol 0.05090g 50.90mg

50 mg / 2 mL = 25 mg/mL

53.7g / X mL = 25 mg/mL

X = 2.15 mL

3) Cool to 55°C
4) add chloramphenicol to LB Agar
5) Plate.

Making LB Agar (Per liter)

- 10g of NaCl
- 10g tryptone
- 5g yeast extract
- 15g Agar
- 1 Liter of deionized H_2O
- Autoclave 1 hr. fluid.

---

test Plates

10 Ng/mL

(50 ng/mL) ⇒ 5mL of nuclease free water.

50mg / 5mL = 10mg/mL

51.7mg / X mL = 10mg/mL

X = 5.17mL.

1) Cool LB Agar to 55°C
2) Add the test into LB Agar
3) Plate

8/1/16

Spinning down primers.

1.) max speed for 5 min (4°C)

2) SR by drote w/ nuclease-free H_2O to total 100 µL.

8/2/16

PCR

For 1 rxn tube (50 µL)

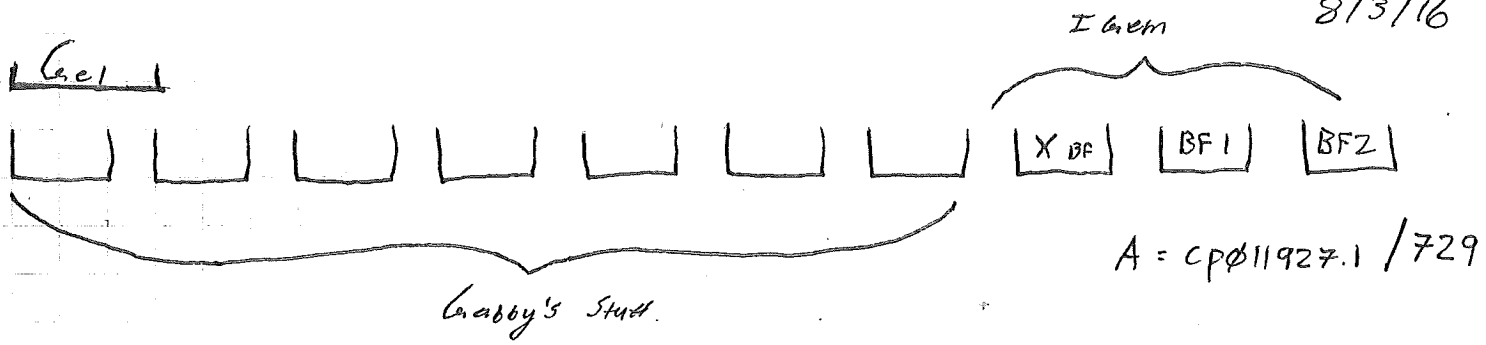
25 µL @5
0.25 µL Forward Primer
0.25 µL Reverse Primer
0.5 µL DNA
24 µL Nuclease-free H_2O

Setting up A and B primers.

A_1	A_2
25 µL @5	25 µL @5
0.25 µL F-A1 Pr1	0.25 µL F-A1 Pr2
0.25 µL Rev Pr	0.25 µL Rev Pr
0.5 µL DNA	0.5 µL DNA
24 µL NF H_2O	24 µL NF H_2O

★ Refer to Fitz's Note book pg. 154

8/3/16

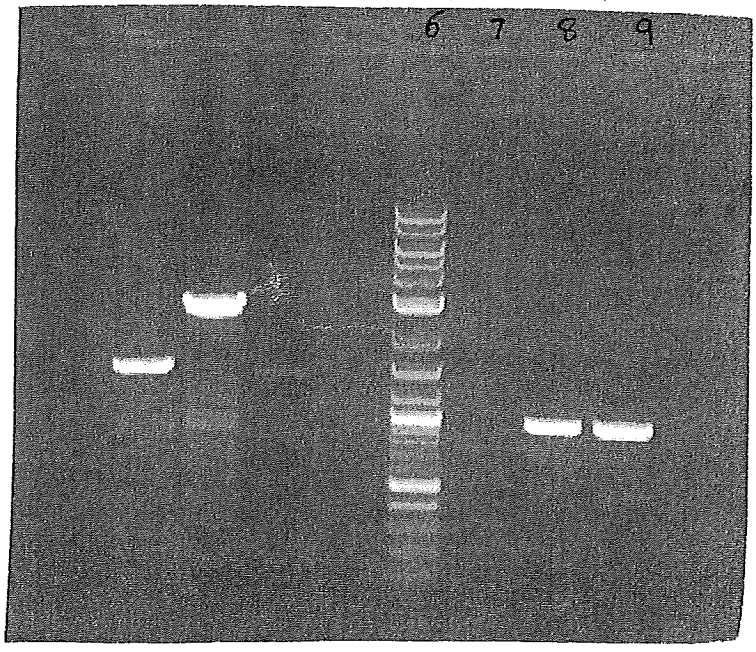


A = cp011927.1 /729

Loading

5 µL of ~~Product~~ primer
1 µL of Purple dye } mix on Parafilm
7 µL of 10 kb ~~Kelly~~ ladder

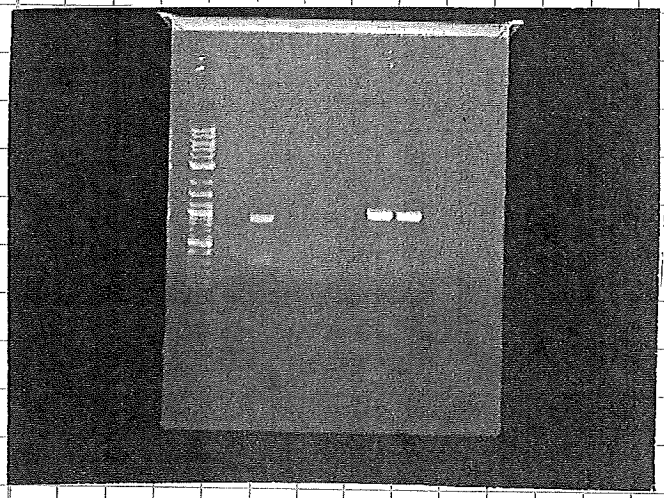
well
6 - 10 kb ladder
7 - XbaF Blank
8 - XbaBF1
9 - BF2



Gel #2

10Kb	X_1	A_1	X_2	A_2	X_B	B1	B2	A_2	
1	2	3	4	5	6	7	8	9	1

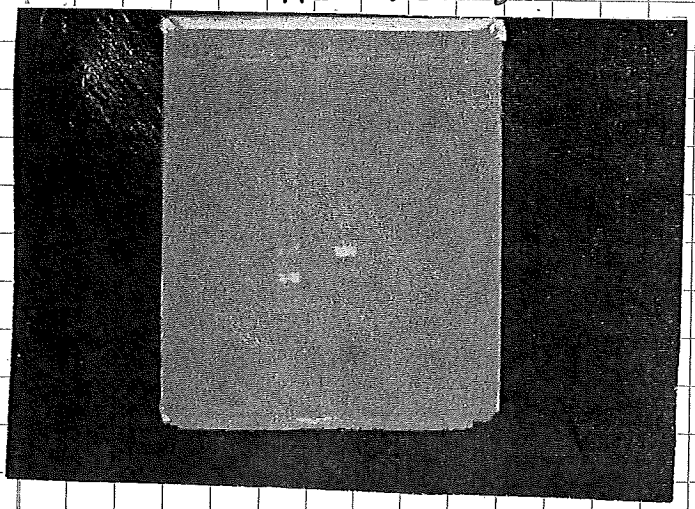
- A gel was run to see if ~~we~~ the sections of DNA we desired existed in Arc A and A+B?
- once we figured out that the DNA we desired existed and showed a strong band, we did a PCR clean up. (See Fitz's lab Notebook)



Ladder, blank a1, a1, blank a2, a2 (which evaporated), blank b, b1, b2, a2 again (still didn't show)

8/4/16 Gel 3

we reran A_2 and X_2 (blank of A2) b/c it evaporated last time.



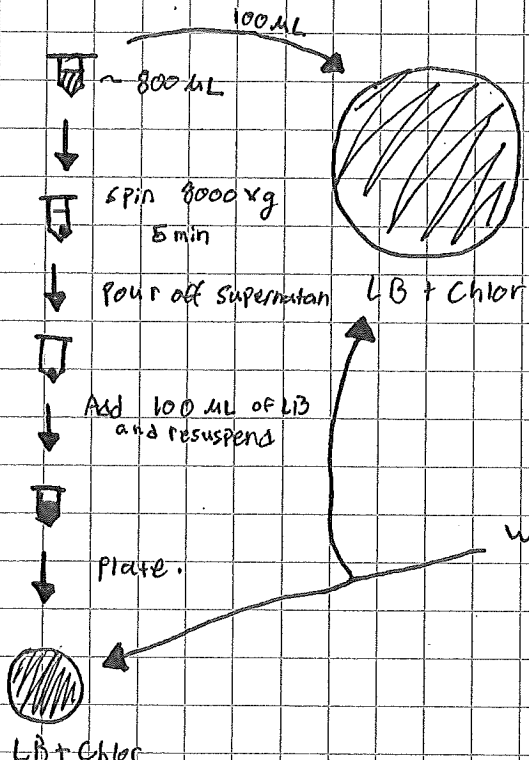
Ladder, blank a2, a2

ligation - refer to fitz's notebook

8/8/1

Transformations

1) Retrieve competent cells from -80 freezer.
2) let it thaw for 30 min in ice.
3) ~~3)~~ After ligation, transformation occurred w/ the following protocol:
3) Remove competent cells from -80°C freezer
4) thaw on ice (20-30 min)
5) add 10pg - 100 ng (1-5 µL) of DNA to 50 µL of competent cells (used 3 µL of each)
6) incubate on ice for 30 min.
7) Heat shock at 42°C for 3 min
8) cool on ice for 5 min
9) Add 750 µL of LB
10) Incubate and Shake for 1 hour
11) warm Selection plates (37°C)
12) Spread 100 µL onto 1 Plate (labeled C-A1 and C-B1) and Spin down the rest @ 8000 x g for 5 min.
13) pour off Supernatant, and resuspend pellet in 100 µL of LB and plate (labeled C-A2 and C-B2)
14) Incubate Plates at 37°C overnight.



6/8/16 Prep for Arc B

Set up for a PCR genes F and H using the high Fidelity Q5 polymerase and 50uL reactions (both will use hevea staggered primers)

F1
25uL Q5
0.25uL Fwd Pr.1
0.25uL rev Pr.
0.5uL DNA
24uL Nuclease Free H_2O

F2
25uL Q5
0.25uL Fwd Pr.2
0.25uL rev Pr.
0.5uL DNA
24uL N-F H_2O

H1
25uL Q5
0.25uL Fwd Pr.
0.25uL Rev. Pr.1
0.5uL DNA
24uL N-F H_2O

H2
25uL Q5
0.25uL Fwd Pr.
0.25uL Rev. Pr2
0.5uL DNA
24uL N-F H_2O

Used the Tm calculator to find the temp for the Q5, which for all 4 is 72°C (F is 1446 bp and H is 1489)

98°C	30 sec
98°C	10 sec
72°C	30 sec
72°C	1:30 sec
repeat	x 34
72°C	3 min
10°C	∞

8/9/16 Gel run for Arc B

10Kb	XH	H1	H2		XF	F1	F2		

- A gel for the F and H gene was run after the PCR. w/ 1 μL of purple dye mixed w/ 5 μL of each component above.

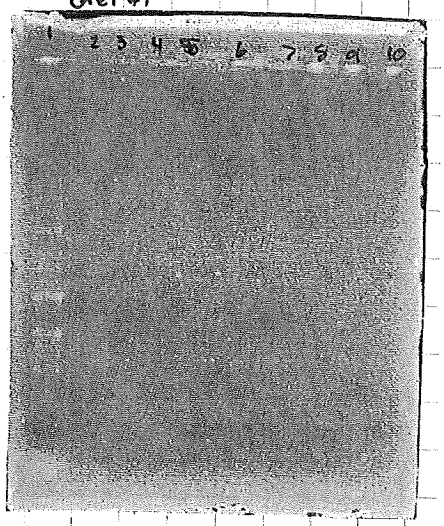
No results, so we reran the gel. this time w/ 1 Kb.

1 Kb

1 μL 1 Kb ladder
4 μL H_2O (N-F)
1 μL dye (purple)

- again no result.

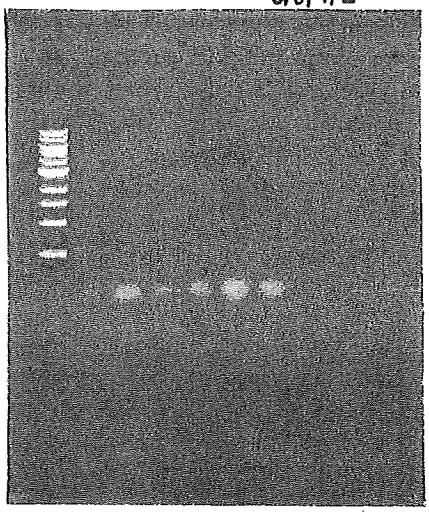
Gel #1



1kb	XH	H1	H2	XF	F1	F2

- b/c No result we re did the PCR (refer to Previous page for PCR procedure.

Gel #2

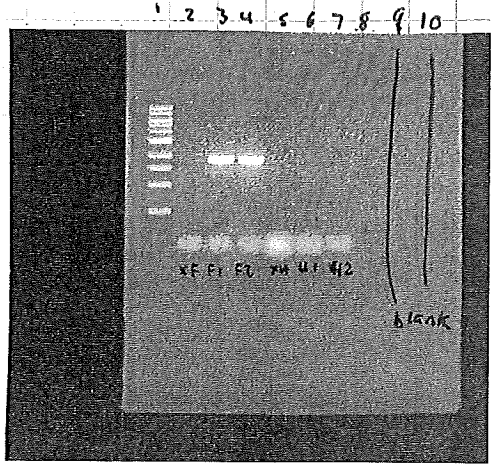


Still zero bands.

↳ must redo PCR. (again)

8/10/16 Gel run for ARC B (attempt #3)

1kb	XF	F1	F2	XH	H1	H2
1	2	3	4	5	6	7



F bands appeared; however, H is still resisting amplification, but since F showed up this time maybe it will work next time?

redo PCR w/ gene H.

- The previous gel run on genes F and H ~~was~~ showed results for only gene F, so we will be PCRing again for gene H ~~and~~ as well as genes A and B because our last 2 transformation produced no results, so we used up all our DNA.

★ NF H_2O = Nuclease-free H_2O.

For A (hetero staggered)

A1

25 µL	Q5
0.25 µL	Fwd Primer 1
0.25 µL	rev primer
0.5 µL	DNA
24 µL	N-F H_2O

A2

25 µL	Q5
0.25 µL	Fwd Pr. 2
0.25 µL	Rev Pr.
0.5 µL	DNA
24 µL	N-F H_2O

tm (71°)

tm (72°) = A1, B, H1, H2

B

25 µL	Q5
0.25 µL	Fwd pr
0.25 µL	Rev pr
0.5 µL	DNA
24 µL	N-F H_2O

H (hetero staggered)

H1

25 µL	Q5
0.25 µL	Fwd Pr
0.25 µL	Rev Pr 1
~~24 µL~~ 0.5 µL	DNA
24 µL	N-F H_2O

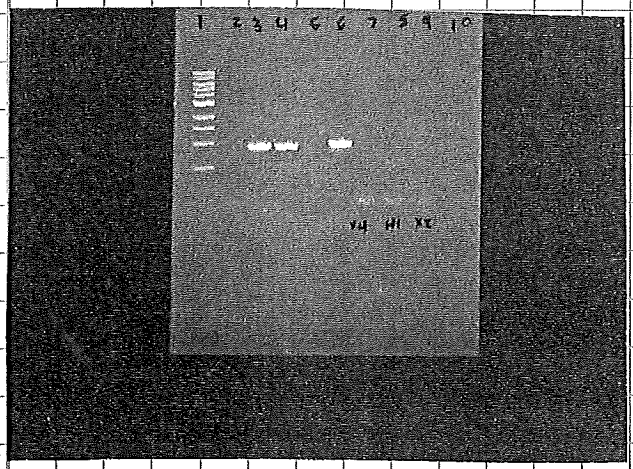
H2

25 µL	Q5
0.25 µL	Fwd pr
0.25 µL	Rev Pr 2
0.5 µL	DNA
24 µL	N-F H_2O

PCR Protocol

	98°C	30 sec.
	98°C	10 sec
gradient →	71°/72°C	30 sec
	72°C	1:30 sec
	repeat	x34
	72°C	3min
	10°C	∞

1K6	XA	A1	A2	XB	B	XH	H1	H2	
	2	3	4	5	6	7	8	9	10



A and B - showed bands; however, H still gave no results.

- run gene H PCR again, but this time use different temperatures.
  - ↳ set up a gradient to experiment w/ multiple different temperatures.

PCR thermalcycler has 8 rows, so we can put 8 different temp.

gradient should be:

72°C back
0
0
0
0
0
0
0
0
65°C front

need to make total of 17 tubes.

1 - blank
8 - H1 label them
8 - H2

XH
$H1_A$ – $H8_A$
$H1_B$ – $H8_B$

8/11/16 PCR again! (only for gene H [arcB])

TH gradient

~~xH~~ H1 - 65°C		H5 - 69.2°C	
H2 - 65.5°C		H6 - 70.7°C	
H3 - 66.4°C		H7 - 71.5°C	
H4 - 67.7°C		H8 - 72.0°C	

H (heterozygous)

HA	x8	HB	x8
25 µL - QS	200 µL	25 µL - QS	200 µL
0.25 µL - Fwd Pr	2 µL	0.25 µL - Fwd Pr	2 µL
0.25 µL - Rev Pr 1	2 µL	0.25 µL - Rev Pr 2	2 µL
0.5 µL - DNA	4 µL	0.5 µL - DNA	4 µL
24 µL - N-F H_2O	192 µL	24 µL - N-F H_2O	192 µL

Blank = ~~50 µL~~ 49.5 µL b/c NO DNA.

↓ total for each tube = 50 µL.

Gel #4

1kb	xH	H_A1	H_A2	H_A3	H_A4	H_A5	H_A6	H_A7	H_A8

No results, however, the agros was very weak, so tomorrow I will re-run the gel w/ fresh Agros.

* ask Brian to see if our genes show up on the Blast.

6/12/16 Cell growth

A 10 µL - No growth
A 100 µL - 16 colonyes.
A 400 µL - 88 colonies.

B 10 µL - 5 colonies
B 100 µL - 43 colonies
B 400 µL - ~~250~~ 150 colonies

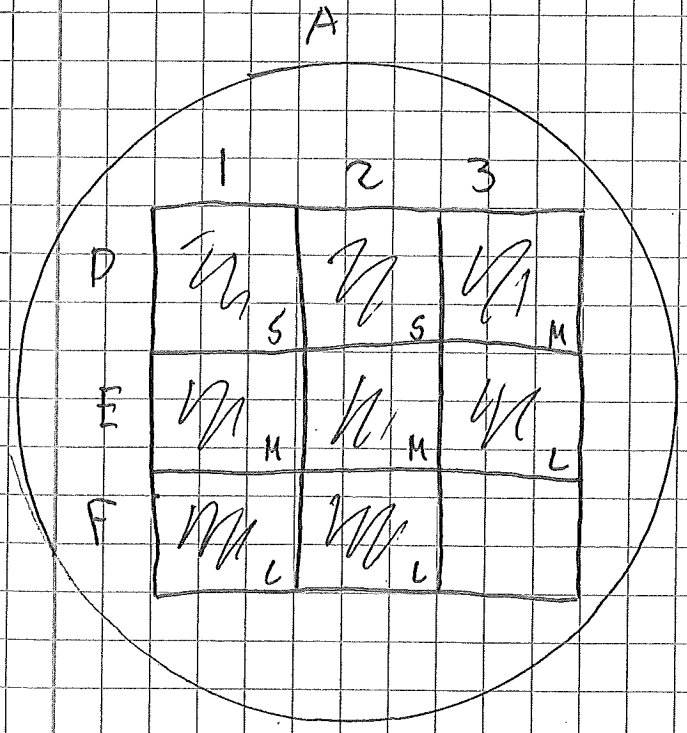
Colony PCR

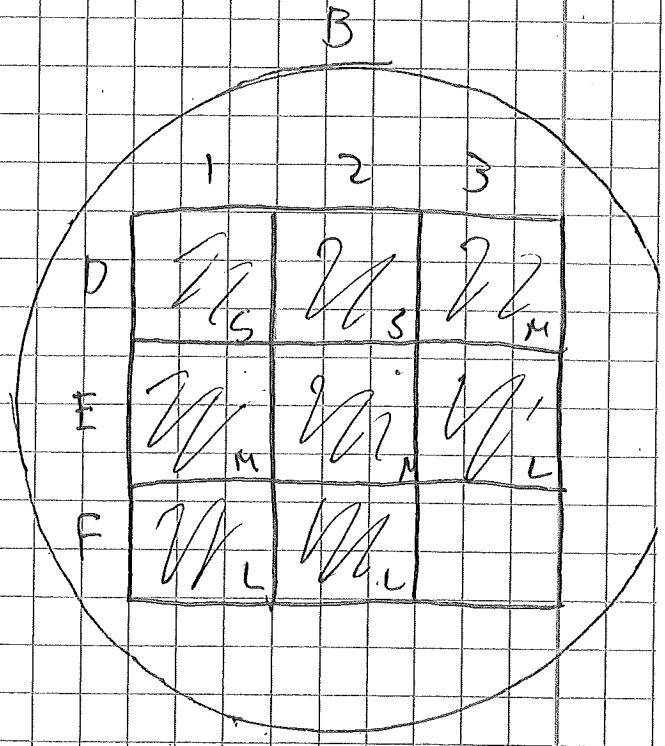
12.5 µL green mix
0.5 µL Primer #1
0.5 µL Primer #2
11.5 µL N-F H_2O
1 colony.

Master Mix

100 µL green mix
~~11~~ µL ~~M13~~ Primer 1
~~11~~ µL Primer 2
~~92~~ ~~84~~ µL N-F H_2O

25 µL per rxn. Put 25 µL in each tube





PCR Protocol

1.) 95° C for 3:00
2) 95° C for 0:30
3) X° C for 0:30 (1-2 °C lower than lowest Tm of primers)
4) 72° C for 1 kb/min (1:00 for colorful Plasmids)
5) 2-4 34X
6) 72° C for 2:00
7) 10 °C ✓

X°C for A = ~~66.8~~ 66.8°C
X°C for B = 67.6°C

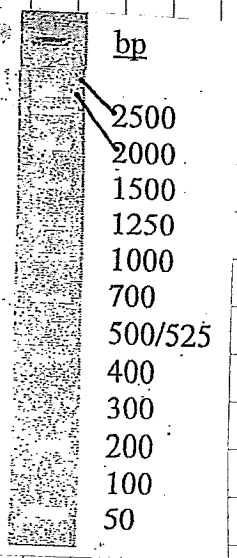
8/12/16 Making Agarose

300mL

294 mL of distilled H_2O
6 mL of 50X TAE
1g / 100mL of agarose for 1%.

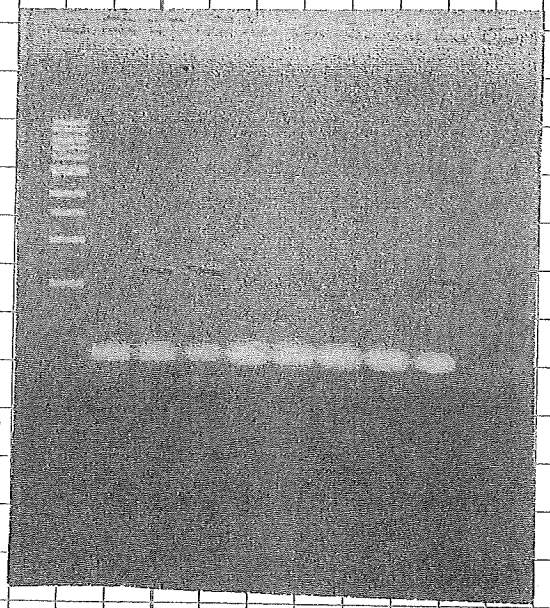
- boil until clear and agarose dissolves.
- cool to "uncomfortably warm"
- add EtBr (toxic) wear gloves. - 15 mL

This is the ladder measurement used for the gel runs from this point forward



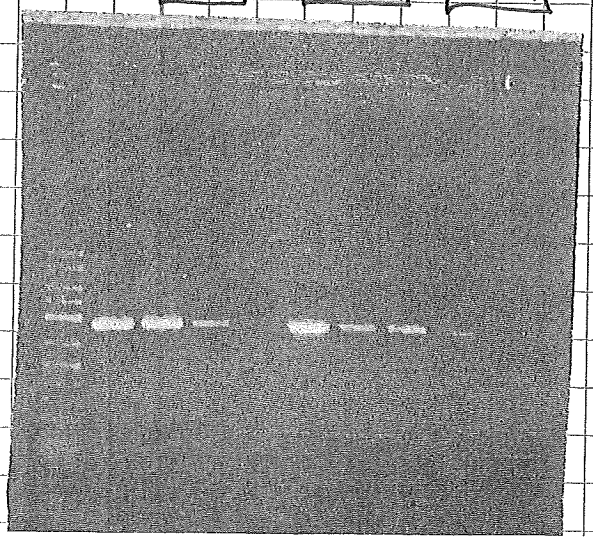
8/15/16 Colony PCR gel run for B

1Kb	$D1_B$	$D2_B$	$D3_B$	$E1_B$	$E2_B$	$E3_B$	F_B	$F2_B$	



- the bands are barely visible. ☆ = bands.
- did not add primers - will re-do.

ladder	$D1_A$	$D2_A$	$D3_A$	$E1_A$	$E2_A$	$E3_A$	F_A	$F2_A$	



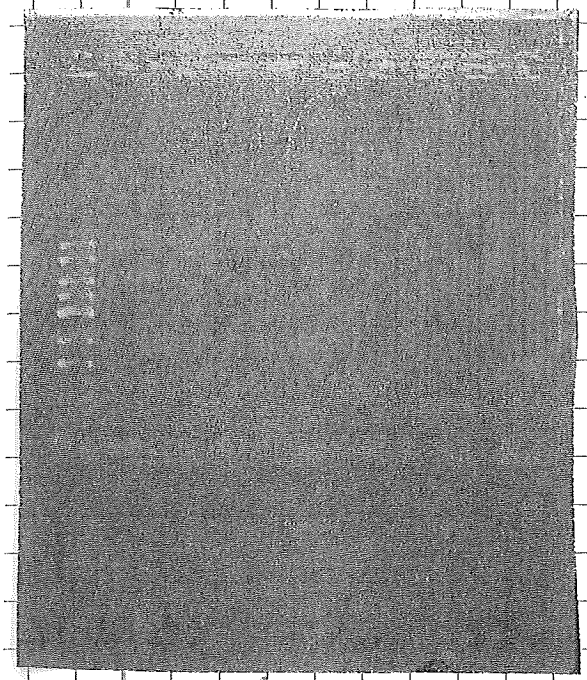
- good clear bands.
- move on to next step.

D1, D2, E2 - best

Second best - ($D3_A$, $F1_A$)

Covel run for gene H (gradient)

Water	H1 A B	H2 A B	H3 A B	H4 A B	H5 A B	H6 A B	H7 A B	H8 A B	HX A B



Still nothing

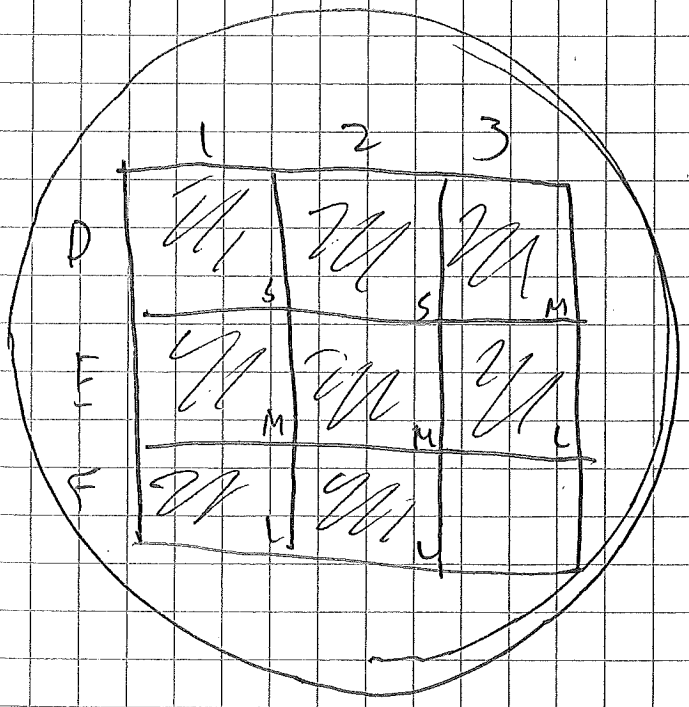
will have to do colony PCR using Nick's Labrenzia colonies.

- Ask Nick for colony.

- Question token, -

what should I do with the grown colonies of gene A and B in the kan vector?

Colony PCR redo for B



Colony PCR → Master Mix

12.5 µL green mix	100 µL green mix
0.5 µL B fwd Pr	4 µL Pr fwd
0.5 µL B Rev Pr.	4 µL Rev Pr.
11.5 µL N-F H_2O	92 µL N-F H_2O.
1 colony.	25 µL in each tube.

PCR Protocol

~~Step 1 ... Step 15.~~

~~Step 3~~ for Step 3 → X °C for B = 67.6 °C − 1 = 66.6 °C ≈ 66 °C

	95 °C	3 min
x34	95 °C	30 sec
	X °C	30 sec
	72 °C	1 min
	72 °C	2 min
	10 °C	∞

6/16/16 PCR clean up

Run PCR clean up for gene F.
clean-up on F1, and F2 (heterostaggered)

F1 - 40 mL
F2 - 40 mL
ratio = 1 (PCR) / 5 (Pb)

$\frac{1}{5} = \frac{40}{x} \Rightarrow 200 = x$

200 mL of Pb -
40 mL of F -

PCR Cleanup →

F1	F2
40 mL F1	40 mL F2
200 mL Pb	200 mL Pb
spin	spin
discard	discard
750 mL PE	750 mL PE
spin	spin
discard	discard
spin	spin
discard	discard
switch to collection tube →	
add 50 mL EB	add 50 mL EB
spin	spin
toss filter	toss filter

Nano drop

	conc. (ng/mL)	260/280	260/230
F1	29.4	2.11	2.92
F2	28.4	2.09	3.65

Since heterostaggered - combine F1 and F2 into single tube label it F

Now a digest will be performed.
get the previously extracted chloramphenicol plasmids from freezer (see Fitz's pg 47)

$\frac{67.7\text{ng}}{1\text{mL}} = \frac{1000\text{ng}}{x\text{mL}}$ 14.8 mL of Plasmid

8/16/16

Recipes for Digest (50 uL rxn)

F1) $\frac{29.4\,ng}{1\,\mu L} = \frac{500\,ng}{x\,\mu L}$ $x = 17.006802721\,\mu L$ of F_1

F2) $\frac{28.4\,ng}{1\,\mu L} = \frac{500\,ng}{x\,\mu L}$ $x = 17.605633803\,\mu L$ of F_2

total = 1000ng.

Since ratio = 1:3 for ligation, make 2 F_1 and F_2 mixtures. 1 for the color vector and one for the kan vector.

Cl Plasmid) $\frac{1000}{3} = 333.333\,ng$ $\frac{67.7\,ng}{1\,\mu L} = \frac{333.33\,ng}{x\,\mu L} \Rightarrow x = 4.923682\,\mu L \approx 4.92\,\mu L$

Plasmid Cl
- 4.92 uL DNA
- 5 uL buffer (cutsmart)
- 1 uL RE1 (EcoR1)
- 1 uL RE2 (Pst1)
- 37.08 uL N-F H_2O

F
- 17 uL of F_1
- 17.61 uL of F_2
- 5 uL of (cutsmart) buffer
- 1 uL of ~~EcoR1~~ (Pst1)
- 9.39 uL of N-F H_2O.

K Plasmid) $\frac{1000}{3} = 333.33\,ng$ $\frac{12.7\,ng}{1\,\mu L} = \frac{333.33\,ng}{x\,\mu L} \Rightarrow x = 26.246719\,\mu L \approx 26.3\,\mu L$

Plasmid K
- 26.3 uL DNA
- 5 uL buffer (cutsmart)
- 1 uL RE1 (EcoR1)
- 1 uL RE2 (Pst1)

needs 333.33ng of K

12.7ng/uL x ? = 333.33ng

x =

No need to digest K, already cut (check Igem box) (it says "Cut")

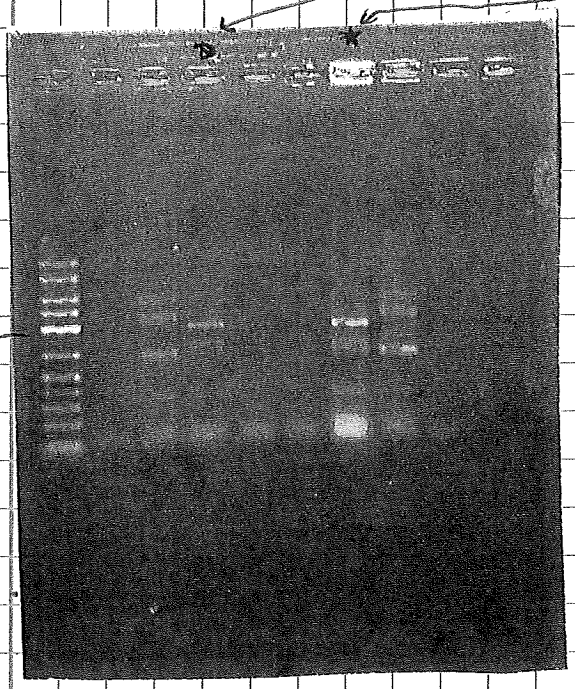
for ligation → 26.3 uL of K

F

look above for Recipe

8/16/16 gel run colony PCR B.

ladder	$D1_B$	$D2_B$	$D3_B$	$E1_B$	$E2_B$	$E3_B$	$F1_B$	$F2_B$	

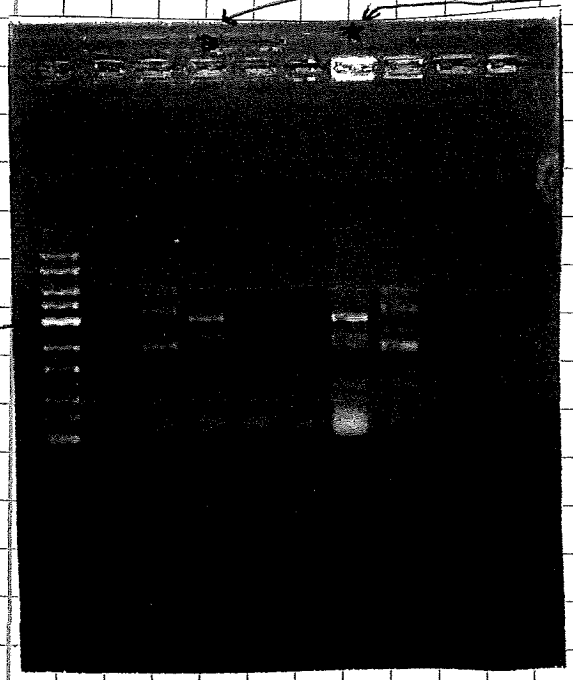


Best band is $E3_B$ colony PCR
Second - $D3_B$ colony PCR.

This gel ran much better than the initial IGem B colony PCR Run.

D — Digestion complete No do a PCR cleanup and place in freezer for ligation and transformation tomorrow.

ladder	$D1_B$	$D2_B$	$D3_B$	$E1_B$	$E2_B$	$E3_B$	$F1_B$	$F2_B$	



Best band is $E3_B$ colony PCR
Second - $D3_B$ colony PCR.

This gel ran much better than the initial IGEM B colony PCR Run.

- Digestion complete. Now do a PCR clean up and place in freezer for ligation and transformation tomorrow.

Nanodrop

	conc (ng/µL)	260/280	260/230
C1	5.2	2.4	1.61
F1	11.4	2.2	1.58
F2	12.4	2.16	1.80

Tomorrow

Ligate
Transform — into both — Kan, Chlor. ✓

7/16 Ligation and transformation.

- heated and slowly cooled the F and C.1 tube to get the correct sticky ends from the heterostaggered PCR. Put F in the thermal cycler for the following.

95°C	5 min.
86°C	20 sec
75°C	30 sec
65°C	30 sec
55°C	30 sec
45°C	30 sec
35°C	30 sec
25°C	30 sec
15°C	30 sec
5°C	∞

Ligate K-F
2 μL T4 buffer
~~Digested Plasmid K (Cut)~~ 2.3 μL of V_K
2.82 μL of F2 (Cut)
11.88 μL ~~of~~ of H_2O
1 μL ligase

After this a ligation is performed for C-F and K-F

Ligate C-F
2 μL T4 buffer
4.8 μL Plasmid C (cut)
3.07 ~~3.82~~ μL F1 (Cut)
9.12 ~~11.6~~ μL H_2O
1 μL ligase.

★ Add ligase Last!!!

Amount of Plasmid needed for 20 μL ligation is 25 ng.

→ (10.8 ng/μL) V_K = 25 ng
V_K = 2.3 μL

Also assumed that since ~35 ng of insert B was needed in Kirstie's notebook, we will still need ~35 ng.

F1 → (11.4 ng/μL) → $\frac{35\text{ ng}}{11.4\text{ ng/μL}}$ = 3.07 μL of F1
F2 → (12.4 ng/μL) → $\frac{35\text{ ng}}{12.4\text{ ng/μL}}$ = 2.82 μL of F2
C1 → (5.2 ng/μL) → $\frac{25\text{ ng}}{5.2\text{ ng/μL}}$ = 4.81 μL of C1

→ for Chlor vector ligation/transformation

for kan vector